From Department of Clinical Neuroscience
Karolinska Institutet, Stockholm, Sweden

INVOLVEMENT OF EPIGENETIC MECHANISMS IN DISEASE INHERITANCE AND PATHOGEGENESIS OF MULTIPLE SCLEROSIS (MS) WITH A FOCUS ON GENOMIC IMPRINTING AND DNA METHYLATION IN $CD4^+$ T CELLS

Sabrina Ruhrmann

Karolinska Institutet

Stockholm 2017

Published by Karolinska Institutet.
Printed by AJ E-print AB

ISBN 978-91-7676-635-4

Involvement of epigenetic mechanisms in disease inheritance and pathogenesis of Multiple Sclerosis (MS) with a focus on genomic imprinting and DNA methylation in $CD4^+$ T cells
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Sabrina Ruhrmann

Principal Supervisor:
Associate Professor Maja Jagodic
Karolinska Institutet
Department of Clinical Neuroscience

Co-supervisor(s):
Professor Fredrik Piehl
Karolinska Institutet
Department of Clinical Neuroscience

Professor Robert A. Harris
Karolinska Institutet
Department of Clinical Neuroscience

Opponent:
Professor Charlotte Ling
Lund University
Department of Clinical Sciences

Examination Board:
Professor Jan Ernerudh
Linköping University
Department of Clinical and Experimental Medicine

Associate Professor Andreas Lennartsson
Karolinska Institutet
Department of Biosciences and Nutrition

Associate Professor Cilla Söderhäll
Karolinska Institutet
Department of Women´s and Children´s health

Für Lars

und

meine geliebten Großeltern

ABSTRACT

Multiple Sclerosis (MS) is a chronic inflammatory and neurodegenerative disease driven by autoreactive $CD4^+$ T cells. Disease etiology is mediated by a strong interplay between genetic and environmental factors implying a role for epigenetic mechanisms. Epigenetics is defined as the study of mechanisms, such as DNA methylation, histone modifications and non-coding RNAs, that result in changes of gene expression without altering the underlying genetic code. Genomic imprinting, one of the most-studied epigenetic marking processes, causes a gene to be expressed only from the maternally or paternally inherited chromosome.

In this thesis we investigate the contribution of epigenetic mechanisms to the etiology and pathogenesis of MS and its animal model, experimental autoimmune encephalomyelitis (EAE).

We investigated the impact of parent-of-origin, in particular genomic imprinting, using two large populations of reciprocal backcross rats and identified that epigenetic mechanisms play a role in EAE inheritance and pathogenesis. Using a transgenic mouse model, we discovered that the imprinted *Dlk1* gene impacts the underlying immune responses in EAE. Further discovery of imprinted genes, using RNA sequencing in adult reciprocal hybrid rats, provided additional insights into the underlying mechanisms of how imprinted genes could interfere with the immune response in EAE by modulating $CD4^+$ T cell function.

Utilizing a genome-wide approach to identify DNA methylation changes between MS patients and controls in $CD4^+$ T cells and monocytes revealed how DNA methylation as an epigenetic mark can impact the function of $CD4^+$ T cells in MS. We identified that DNA methylation acts as a mediator of the major MS risk factor, the *HLA-DRB1* gene, to impact expression of the HLA class II molecules that present antigens to $CD4^+$ T cells. DNA methylation further affected $CD4^+$ T cells directly through changed epigenetic marking of a microRNA, miR-21, impacting miR-21 expression and its target genes.

Our findings collectively underline the importance of integrating multiple layers of gene regulation to identify novel mechanisms involved in the etiology and pathogenesis of complex diseases like MS. This will in turn open up for novel therapeutic approaches based on targeting dysregulated epigenomes in human disease.

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LIST OF SCIENTIFIC PAPERS

I. **Parent-of-origin effects implicate epigenetic regulation of experimental autoimmune encephalomyelitis and identify imprinted *Dlk1* as a novel risk gene**

Stridh P*, Ruhrmann S*, Bergman P, Thessén Hedreul M, Flytzani S, Beyeen AD, Gillett A, Krivosija N, Öckinger J, Ferguson-Smith AC, Jagodic M

PLoS Genetics. 2014 Mar 27;10(3)

*Authors contributed equally

II. **A survey of imprinted genes in CD4$^+$ T cells and immune tissues in the rat: implications for immune mediated diseases**

Ruhrmann S, N´Diaye M, Bergman P, Needhamsen M, Hoekstra-Wakker K, Spierings, D, Bevova M, Piket E, Guryev V, Jagodic M

Manuscript

III. **Next generation sequencing identifies microRNAs that associate with pathogenic autoimmune neuroinflammation in rats**

Bergman P, James T, Kular L, Ruhrmann S, Kramarova T, Kvist A, Supic G, Gillett A, Pivarsci A, Jagodic M

Journal of Immunology. 2013 Apr 15;190(8):4066-75

IV. **Hypermethylation of *MIR21* in CD4$^+$ T cells from patients with relapsing-remitting Multiple Sclerosis associates with lower miR-21 levels and cocomitant up-regulation of its target genes**.

Ruhrmann S, Ewing E, Piket E, Kular L, Cetrulo-Lorenzi, J C, Fernandes S J, Morikawa H, Aeinehband S, Sayols-Baixeras S, Aslibekjan S, Absher D M, Arnett D K, Tegner J, Gomez-Cbrero D, Piehl F, Jagodic M

Manuscript

V. **DNA methylation at HLA as a mediator of the risk for Multiple Sclerosis by *DRB1*15:01* and novel genetic variants**

Kular L*, Liu Y*, Ruhrmann S, Gomez-Cabrero D, Zheleznyakova G, Aeinehband S, James T, Lindén M, Górnikiewicz B, Stridh P, Link J, Dilthey A T, Olafsson S, Eggertsson HP, Halldorsson BV, Celius EG, Søndergaard HB, Tegnér J, Harbo HF, Oturai AB, Jonsdottir I, Stefansson K, Olsson T, Piehl F, Ekström TJ, Kockum I*, Feinberg AP*, Jagodic M*

Manuscript

* Authors contributed equally

ADDITIONAL PUBLICATIONS

The author of this thesis has also contributed to the following papers

I. **Genomic imprinting: A missing piece of the Multiple Sclerosis puzzle?**
Ruhrmann S, Stridh P, Kular L, Jagodic M
Int J Biochem Cell Biol. 2015 Oct;67:49-57

II. **Functional genomics analysis of vitamin D effects on CD4+ T cells in vivo in experimental autoimmune encephalomyelitis**
Zeitelhofer M, Adzemovic MZ, Gomez-Cabrero D, Bergman P, Hochmeister S, N'diaye M, Paulson A, Ruhrmann S, Almgren M, Tegnér JN, Ekström TJ, Guerreiro-Cacais AO, Jagodic M
Proc Natl Acad Sci U S A. 2017 Feb 28;114(9):E1678-E1687.

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Assadi G, Vesterlund L, Bonfiglio F, Mazzurana L, Cordeddu L, Schepis D, Mjösberg J, Ruhrmann S, Fabbri A, Vukojevic V, Percipalle P, Salomons FA, Laurencikiene J, Törkvist L, Halfvarson J, D'Amato M
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J Leukoc Biol. 2016 Mar;99(3):437-46.

V. **Interferon-beta affects mitochondrial activity in CD4+ lymphocytes: Implications for mechanism of action in multiple sclerosis**
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Epigenetics. 2013 Mar;8(3):333-46.

VII. **Functional energetics of CD4+-cellular immunity in monoclonal**

antibody-associated progressive multifocal leukoencephalopathy in autoimmune disorders

Haghikia A, Perrech M, Pula B, Ruhrmann S, Potthoff A, Brockmeyer NH, Goelz S, Wiendl H, Lindå H, Ziemssen T, Baranzini SE, Käll TB, Bengel D, Olsson T, Gold R, Chan A

PLoS One. 2011 Apr 20;6(4):e18506.

CONTENTS

LIST OF ABBREVIATIONS

AGO	Argonaute
APC	antigen presenting cell
BBB	blood-brain-barrier
CNS	central nervous system
CSF	cerebrospinal fluid
DMD	disease modifying drug
DMR	differential methylated region
DNMTs	DNA methyltransferases
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
EDSS	expanded disability status scale
EWAS	epigenome-wide association study
GM-CSF	granulocyte macrophage – colony stimulating factor
HATs	histone acetyltransferases
HDACs	histone deacetylases
HLA	human leukocyte antigen
ICR	imprinting control region
lncRNA	long non-coding RNA
MHC	major histocompatibility complex
miRNA	microRNA
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
ncRNA	non-coding RNA
NGS	next generation sequencing
pri-miRNA	primary miRNA
PP-MS	primary progressive – MS

RISC	RNA induced silencing complex
rMOG	recombinant MOG
ROS	reactive oxygen species
RR-MS	relapsing remitting - MS
rRNA	ribosomal RNA
SNVs	single nucleotide variations
SP-MS	secondary progressive - MS
TCR	T cell receptor
Th	T helper
TNF	tumor necrosis factor
Treg	T regulatory
UTR	untranslated region
VLA4	very late antigen 4

1 INTRODUCTION

1.1 Immune System

The immune system is a defense system that protects against disease by recognizing and fighting a variety of foreign agents while distinguishing them from the body's own healthy tissue. The immune system consists of two parts, the innate and the adaptive immune system.

Whereas the innate immune system comprises immune cells and mechanisms (such as neutrophils, monocytes, macrophages, complement system, cytokines and acute phase proteins) that allow for a rapid but non-specific response, the adaptive immune system is highly specific for a particular antigen that triggers a response of lymphocytes and it takes days or even weeks to develop[1]. There are two broad classes of the adaptive immune responses, antibody and cell-mediated responses that are carried out by B and T lymphocytes, respectively.

In the cell-mediated adaptive immune response, cytotoxic $CD8^+$ T cells attack infected or damaged cells directly, while 'helper' $CD4^+$ T cells regulate the adaptive immune response by directing other immune cells to perform various tasks. To exert their effector functions, naïve T cells need to be activated by antigen presenting cells (APCs) such as dendritic cells, monocytes, macrophages and B cells that express major histocompatibility complex (MHC) molecules that present the antigen to T cells and at the same time provide co-stimulatory signals. $CD4^+$ and $CD8^+$ T cells express T cell receptors (TCR) that recognize antigens presented by MHC class II molecules and MHC class I molecules, respectively[1].

Several types of helper $CD4^+$ T cell lineages, including T helper (Th) 1, Th2, Th17 and regulatory T cells (Treg), can be induced depending on the interaction with APCs and the type of cytokines they are exposed to. The subsets are characterized by the cytokines they produce and functional differences that include the attraction of cells of the innate immune system[1].

Malfunctioning of the immune system can cause pathological conditions such as autoimmune diseases in which the immune system fails to distinguish between self and non-self and starts attacking body's own healthy tissue.

1.2 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) affecting mostly young adults, especially women[2]. The disease, depending on which area of the CNS is affected can display a variety of symptoms with the most common being sensory loss, visual disturbance, motor weakness and impaired balance[2]. Most MS patients initially present with the relapsing-remitting form of MS (RR-MS), which is characterized by episodes of active disease (relapse) and periods of clinical inactivity (remission). Within 20 years from diagnosis, a majority of RR-MS patients will convert to a secondary-progressive form (SP-MS) characterized by a continuous worsening with or without overlaid clinical relapses. In 10% of patients the disease is progressive from the onset and it is classified as a primary-progressive MS (PP-MS)[2].

Accurate diagnosis based on clinical symptoms, accessed by the expanded disability status scale (EDSS)[3], and magnetic resonance imaging (MRI) findings is essential to allow for early interference with the disease course[4].

1.2.1 Treatment

Over the last 20 years the approval of novel disease modifying drugs (DMDs) has led to the revolution of the therapeutic field in MS. With the release of oral DMDs there is now actually a choice to injectable DMDs when considering first line treatments.

Recombinant interferon beta (Rebif, Avonex) was the first drug approved for RR-MS in 1993[5]. It mediates an anti-inflammatory immune response and needs to be administered by self-injections every two weeks. Non-transient side effects observed following interferon beta treatment were irritations at the sites of injection. However, some patients develop neutralizing antibodies against the treatment[6]. With the approval of natalizumab as a second line treatment in 2006[7] a good choice seemed to be available for patients where first line treatments failed. As a humanized monoclonal antibody that binds to alpha-4 integrin of very late antigen 4 (VLA4), a surface marker present on immune cells, natalizumab prevented leucocytes from migrating across the blood-brain-barrier (BBB)[7]. Unfortunately, patients may develop severe side effects namely progressive multifocal leukoencephalopathy, a potentially lethal opportunistic brain infection caused by the JC virus[8].

The first oral drug developed and released was Fingolimod (Gilenya). Fingolimod targets the sphingosine 1 phosphate receptor (S1PR1) and its main mechanism is the sequestration of lymphocytes in the lymph nodes due to S1PR1 block[9]. Side effects depend on the distribution of the receptor, which can be also found in heart, retina, lung and liver, and can in the worst case lead to heart block[10].

In addition to the above described DMDs several monoclonal antibodies targeting the CD20 antigen on B cells have emerged on the market, namely rituximab, ocrelizumab and ofatumumab[11]. Following the treatment with rituximab, B cells are depleted and decreased levels of cytokines and T cells can be observed in the CSF[13]. Further, the antigen presentation

and co-stimulation ability of B cells is impaired after CD20 treatment[14]. No severe side effects have been observed.

The wide range of treatments available for the relapsing-remitting form of MS, allows clinicians nowadays a more personalized treatment approach, to balance risks against benefits of different treatments for every single patient[15]. However, all effective treatments target the immune system in a broad manner. Despite the expanding range of treatments for patients displaying a relapsing-remitting disease course, treatment of the progressive forms of MS still remains a challenge and further investigations are needed.

1.2.2 Pathogenesis

MS is considered to be an autoimmune disease that is driven by $CD4^+$ T cells. This hypothesis originates from its similarities in pathogenesis with the animal model of MS, experimental autoimmune encephalomyelitis (EAE) that is known to be driven by myelin specific T cells (described in more detail below). These myelin autoreactive T cells have been found in MS patients but also in healthy controls with no differences in frequencies. However, autoreactive T cells in MS patients were observed to be more activated compared to the T cells found in controls[106]. Moreover, genetic studies have identified Human Leukocyte Antigen (HLA) class II alleles that present antigen to $CD4^+$ T cells as the major risk alleles for developing MS[18,29].

The exact mechanism how this activation of $CD4^+$ T cells in the periphery takes place remains still poorly understood and needs to be further investigated. However, the current opinion in the field suggests mechanisms like molecular mimicry where T cells generated against a foreign antigen cross-react with self-antigens with a similar sequence or that T cells become activated due to myelin antigens that are constantly present in the peripheral lymph nodes[106].

Activated $CD4^+$ T cells together with activated B cells and monocytes enter the CNS across the BBB. A crucial mechanism throughout this migration is the interaction between the VLA4 adhesion molecule on leucocytes and Vcam1 on endothelial cells, which allows for the leucocytes to overcome the immune privileged BBB. The importance of this step is further underlined by the observation that the migration is inhibited after antibodies against VLA4 have been administered[106].

In the CNS, $CD4^+$ T cells become reactivated by APCs presenting their target antigen and start to differentiate into their various helper subsets, which exert different effector functions. Together with macrophages and microglia, they secrete proinflammatory cytokines, e.g. IL-17 and IFNγ that trigger the axonal demyelination process by attracting further innate and adaptive immune cells[106].

Beside $CD4^+$ T cells there is also emerging evidence for $CD8^+$ T cells to be involved in MS pathogenesis mediating axonal damage by the secretion of cytokines and cell contact mediated lysis. $CD8^+$ T cells are more prominent in MS lesions than $CD4^+$ T cells[17] and

genetic studies have identified HLA class I alleles that present antigen to $CD8^+$ T cells as risk alleles[18].

Further clonal expanded B cells and oligoclonal bands in the CSF of MS patients and antibodies directly targeting the axonal myelin sheaths imply also a crucial role for B cells in MS pathogenesis. These findings are further strengthened by the successful treatment with disease modifying drugs that target the CD20 antigen on B cells[106].

1.2.3 Experimental Autoimmune Encephalomyelitis

To gain insights into the immune responses underlying MS, animal models are essential since the reactions in the CNS tissue are difficult to study in humans. The most commonly used animal model for MS is EAE[19].

In EAE, immunization with CNS antigen in Freud´s adjuvant results in the generation of pathogenic $CD4^+$ T cells, mainly of the Th1 and Th17 type, in peripheral lymphoid organs[16]. Pathogenic $CD4^+$ T cells migrate to the CNS where they become reactivated by macrophages, dendritic cells and B cells presenting the autoantigen. Reactivated $CD4^+$ T cells start to secrete the cytokines such as IL-17, IFNγ, tumor necrosis factor (TNF) and granulocyte macrophage – colony stimulating factor (GM-CSF). Secretion of IL-17 leads to the further release of cytokines, chemokines and metalloproteases by local tissue cells mediating the further breakdown of the BBB and attraction of monocytes and neutrophils[21]. In addition to IL-17, GM-CSF also recruits neutrophils to the site of inflammation and is implied to influence monocytes and their impact on Th differentiation[21]. Further, IFNγ and TNF stimulate myeloid effector cells such as inflammatory monocytes, macrophages and neutrophils and lead to the damage of myelin by reactive oxygen species (ROS)[21]. In the peripheral lymph nodes or in the CNS, activated B cells that have become antibody-secreting plasma cells or plasma blasts may also release myelin targeting antibodies[21]. The ability to induce MS-like pathology by adoptive transfer of $CD4^+$ T cells to naïve recipients further supports an important role of $CD4^+$ T cells in disease pathology[20].

EAE can be induced and studied in a variety of animal species from rodents to non-human primates[19]. One of the most commonly used CNS antigens is a myelin oligodendrocyte glycoprotein (MOG), which is a minor component of the myelin but expressed on the outer layer and capable of eliciting both antibody and cell-mediated immune response. Immunization of the C57BL/6 mouse strain with extracellular portion of recombinant MOG (rMOG) in complete Freud´s adjuvant, containing Mycobacterium tuberculosis, induces EAE with a progressive course that resembles human disease[19]. The model requires additional Bordetella pertussis toxin injections that are known to further permeabilize the BBB[22]. Immunization of the DA rat strain with rMOG in incomplete Freud´s adjuvant results in a relapsing-remitting form of EAE that shares numerous features with MS[23].

Despite differences compared to the human counterpart, several approved therapies for MS have been developed in EAE, demonstrating its utility when appropriately applied and interpreted.

1.2.4 Risk factors for Multiple Sclerosis

A growing body of evidence suggests that MS results from an interplay between genes and environmental factors. Evidence for a family aggregation of MS was provided by the observation that first degree relatives have a greater risk of developing MS than the rest of the population implying genetic factors in the pathogenesis of MS[25,26]. Further proof for an involvement of genetics came from studies conducted in adoptees and twins. Adoptees with MS and individuals having affected adoptive family members do not differ in their risk to develop MS from the rest of the population[27]. Monozygotic twins show a higher concordance rate for MS compared to dizygotic twin pairs[26,28]. The single most prominent risk for MS maps to the HLA locus and it associates, more specifically, with the *HLA-DRB1*15:01* allele[18]. The underlying mechanism is likely associated with the binding and presentation of antigens to $CD4^+$ T cells by the HLA class II molecules. Beside the HLA locus more than 100 risk loci have been established to associate with MS[29]. Contrary to the HLA locus non-HLA risk loci exert only modest individual effects but in regards to their biological functions point to immune related functions such as lymphocyte proliferation and Th differentiation[18]. However, both the rather low familial recurrence rate and a concordance rate of at best only 25% in monozygotic twins indicate the involvement of other factors in the development of disease.

Environmental factors that most consistently associate with the risk of developing MS include Epstein-Barr virus (EBV) infection, lack of vitamin D/sun exposure and cigarette smoking[30]. Individuals having encountered an EBV infection during their lifetime displayed a higher risk to develop MS compared to individuals that had not encountered the virus[31]. In addition, risk of MS is also known to be associated with the lack of vitamin D/sun exposure[32,33]. Studies showed that females who received vitamin D supplement displayed a lower risk to develop disease than females who did not get the supplement [34]. In 2009, Hedström *et al.* provided convincing evidence for smoking being an important environmental factor in the development of MS disease by showing that MS risk was 50% higher in ever smokers when compared to never smokers[35.]

Interestingly, a strong gene-environment interaction has been demonstrated for smoking and the *HLA-DRB1*15:01* risk haplotype. Individuals carrying the risk allele that also had a history of tobacco smoking displayed a 14-fold higher MS risk compared to non-smokers and non-carriers of *HLA-DRB1*15:01*[36].

1.3 Epigenetics

In the year of 1942 Waddington coined the term epigenetics as "changes that occur in the phenotype without altering the genotype" [37,38].

Today epigenetics is described as the study of mechanisms that impact gene expression without changing the actual underlying DNA sequence[39]. DNA methylation, histone modifications, polycomb complexes and non-coding RNAs (ncRNAs) are the main studied epigenetic mechanisms that influence gene expression in a tissue and cell type specific manner[40]. Acquired epigenetic changes can be stably inherited through mitosis, but the extent of potential trangenerational inheritance in humans remains unknown. Environmental factors like diet, smoking or physical activity can induce epigenetic changes[41,42,43], which are also under strong regulation by the genetic background[44]. Knowing that most of the known complex diseases result from an interplay between environment and genetics[45] and that genetics only explains a part of disease risk ('hidden heritability') makes it tempting to speculate that epigenetic mechanisms can be the missing link in the etiology of these diseases.

In this thesis we mainly studied genomic imprinting, a well-known epigenetic marking process that can cause parent-of-origin effects, and DNA methylation, a well-known epigenetic mechanism, in the context of MS and its animal model.

1.3.1 Genomic Imprinting

Genomic imprinting describes an epigenetic marking process that causes a gene to be expressed only from the maternally or paternally inherited chromosome[46].

The term Genomic Imprinting was first coined in the 1960s by Helen Crouse describing the elimination of paternally derived X chromosomes in sciarid flies[47]. In 1984, Solter and Surani delivered the defining experiments, demonstrating that in mammals not all genes are expressed from both inherited chromosomes in the same nucleus, but the underlying mechanisms remained still unknown[48]. Six years later the first imprinted genes, *Igf2r, Igf2, H19,* were identified[49,50,51,52] and in 1993 Jaenisch and co-workers provided the first evidence for DNA methylation being one of the underlying epigenetic mechanisms in genomic imprinting[53]. Up to date approximately 150 imprinted genes in mice and 100 imprinted genes in humans have been identified and well established[54,55,56,57].

Imprinted genes tend to occur in clusters of 3-12 genes that can spread over 80 kb to 3.7 Mb of DNA[46] (Fig.1). All imprinted gene clusters contain a differentially methylated region (DMR) that partly controls the imprinting of the cluster and is therefore also described as the imprinting control region (ICR)[58,59,60,61,62]. Beside the ICR, long non-coding RNAs (lncRNAs)[63,64,65] and insulators like the zinc finger protein CTCF[66,67] associated with different imprinted clusters are also involved in the regulation of imprinting. Over-expression of these lncRNAs due to methylation changes in the ICR of imprinted gene clusters is a common feature for imprinting disorders like the Beckwith-Wiedemann syndrome[68]. This

indicates the importance of the interplay between ICR and lncRNAs for the regulation of imprinted mRNA genes in the clusters. Further, genomic imprinting can differ between individuals and change with age, tissue or even cell type suggesting diverse mechanisms of regulation[56,57].

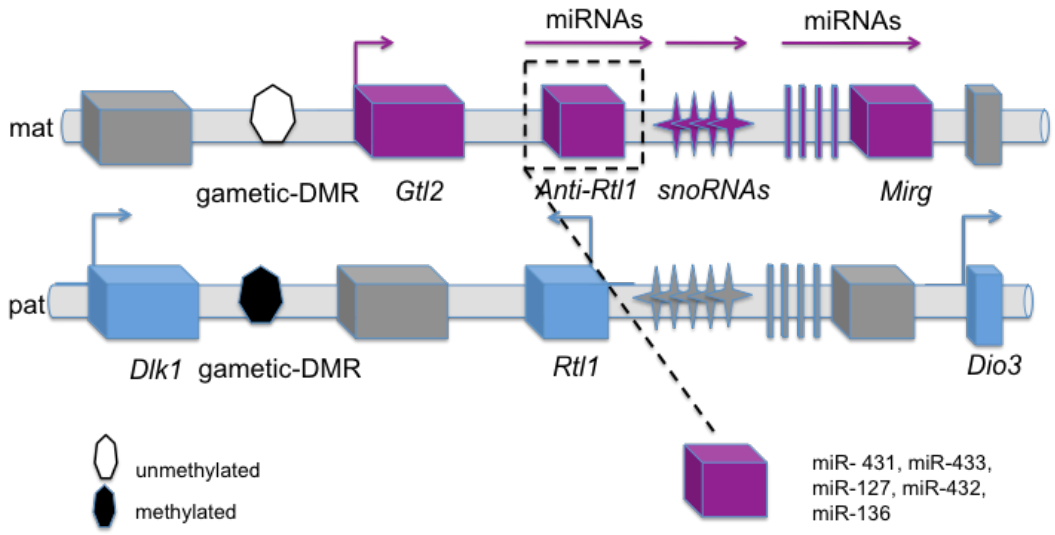

Figure 1: Imprinted *Dlk1-Dio3* locus on rat chromosome 6

Imprinted genes have important functions in regulating fetal growth and development in mammals. Here, paternally expressed imprinted genes function as growth promoters whereas maternally expressed imprinted genes function as growth repressors[46]. Besides their role in development, several imprinted genes have been implicated in regulation of immune responses, including differentiation and activation of T and B lymphocytes that constitute the major cellular components of the adaptive immune response[69].

Why genomic imprinting evolved in mammals remains still under investigation but two attractive hypotheses are intensively discussed in the field:

"Genomic Imprinting evolved in response to a parental conflict situation" by Moore and Haig 1991[70].

The hypothesis by Moore and Haig is based on the opposite interests of the paternal and maternal genome. Namely, whereas paternally expressed imprinted genes are known to promote embryonic growth trying to maximize the chance for a single individual carrying a specific paternal genome, maternally expressed imprinted genes inhibit embryonic growth with the purpose to divide their genome to several embryos possibly carrying different paternal genomes.

"Trophoblast defense" by Varmuza and Mann 1994[71].

The "Trophoblast defense" hypothesis formulated by Varmuza and Mann in 1994 is on the contrary founded on the fact that the maternal genome allows for internal reproduction by females having the necessary anatomically requirement whereas males lack such an

anatomical equipment. Genomic Imprinting is therefore supposed to silence genes on the maternal chromosome that would increase placental growth or activate genes that counteract the process.

Both hypotheses are not able to explain the full extent of data available on genomic imprinting[72].

Genomic imprinting is one of the best-characterized epigenetic mechanisms that cause parent-of-origin effects. The term parent-of-origin effect refers to the phenomenon in which a disease phenotype of the predisposing allele depends on the parental origin, *i.e.* on whether the allele was inherited maternally or paternally. Beside genomic imprinting, additional mechanisms causing parent-of-origin effects involve the sex chromosomes, mitochondria, gender transmission bias, and trans-generational effects (including maternal intrauterine effects and maternal-offspring interactions)[73].

1.3.2 DNA methylation

"5mC as the 5th base of the genetic code" by David Allis[74] .

DNA methylation (5mC) is described as a mechanism where a methyl group (CH3) is added at the 5' position of the pyrimidine ring of the cytosine residues in CpG dinucleotides mediated by DNA methyltransferases (DNMTs).

The actual existence of chemical modifications of DNA bases, like for instance the addition of a methyl group to a cytosine, was discovered in 1948[75]. Thirty years later Razin and Bird provided experimental evidence for the functional impact of DNA methylation on gene expression and the existence of CpG islands, respectively[76,77]. Studying the impact of methylation changes in the living cell became possible with the discovery of the nucleoside analogue, 5-azacytidine, which inhibits DNA methylation[78].

CpG islands (CGIs) describe the accumulation of CpG residues within a region of 1 kb that can occur upstream of promoters and in general appear unmethylated[79]. However, during the process of X chromosome inactivation in female mammals those unmethylated CpG islands become de novo methylated mediating the repression of gene transcription on the X chromosome undergoing inactivation[80]. De novo methylation is regulated by the methylation enzymes DNMT3A and DNMT3B[81] and maintained by the methyltransferase DNMT1[82]. Methylation occurring in promoter regions can lead, as described for the process of X chromosome inactivation, to repression of gene transcription, either by directly inhibiting the binding of transcription factors or indirectly by recruiting methyl CpG binding proteins[83,84].

DNA methylation is involved in general processes like the aforementioned processes of X chromosome inactivation, genome stability and genomic imprinting. However, DNA methylation also has a critical role in immune cell specific functions, like $CD4^+$ T helper cell differentiation and T cell activation[85,86,87,88]. Therefore alterations in DNA methylation,

triggered by environmental risk factors like smoking, or aging or genetic risk factors, might lead to aberrant $CD4^+$ T cell function due to changes in gene expression but also incomplete X chromosome inactivation and disturbances in genomic imprinting.

1.3.3 Histone modifications

In the nucleus, 147 base pairs of DNA are wrapped around dimers of the histone proteins H2A, H2B, H3 and H4 forming the basic unit of chromatin, the nucleosome[89].

Tails of histone proteins, which are rich in arginine and lysine residues, are prone to posttranslational modifications that subsequently can lead to changes in gene expression[90]. Lysine acetylation and deacetylation of histone proteins associate with gene transcription and gene repression, respectively[90]. Enzymes catalyzing the process are known as histone acetyltransferases (HATs) and histone deacetylases (HDACs)[91]. Further, methylation of lysine residues mediated by histone lysine methyltranferases can associate with both gene transcription and repression[90]. The first histone lysine demethylase was discovered in 2004[92].

Variation in the histone proteins H2A or H3 also allows for modification of the chromatin structure and is associated with processes like cell proliferation[93] and CNS development[94].

1.3.4 Non-coding RNAs

"80% of the genome is transcribing ncRNAs", ENCODE[95].

Non-coding RNAs describe RNA transcripts that do not encode for proteins. With the invention of high throughput sequencing, evidence was provided for the existence of a higher number of genes that encode for non-coding transcripts than the number of genes for protein-coding transcripts[95].

With the identification of messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA), RNA was first believed to only act as a template for protein synthesis and that the increased number of non-coding transcripts were rather debris or noise than of functional importance[96].

However, there is emerging evidence for roles of ncRNAs including small RNAs like microRNAs (miRNAs) and longer non-coding transcripts (>200 nt) going far beyond the role of RNA first described.

1.3.4.1 MicroRNAs

MicroRNAs are non-coding RNAs that are short (20-23 nt), single stranded and processed from hair pin precursors. Their discovery in 1993 and their functional impact on gene regulation provided further evidence for RNAs being more than only a template for DNA[97,98].

Primary miRNA hairpins (pri-miRNA) are produced by RNA polymerase II and cleaved by a microprocessor complex containing the enzyme Drosha in the nucleus. After cleavage, Exportin 5 exports the pre-miRNA from the nucleus into the cytoplasm. Here, the enzyme

Dicer cleaves the pre-miRNA to its mature length, which results in the mature miRNA being loaded into the RNA induced silencing complex (RISC) together with Argonaute (AGO) proteins. By base pairing between the seed region (2-8 nucleotides at the 5' end of the mature miRNA) of the miRNA and the 3' untranslated region (3'UTR) of the target gene the mature miRNA guides the RISC to silence the target mRNA either by degradation, translational repression or deadenylation[99].

With their regulation of multiple target mRNAs[100], miRNAs have been demonstrated to be involved in the regulation of many physiological, developmental and disease processes. Among other developmental processes miRNAs have been demonstrated to be crucial for the development of the immune system but also in particular for the innate and adaptive immune response[101,102]. In T and B cells impaired miRNA biogenesis leads to aberrations in their development and T helper cell differentiation[102].

1.3.4.2 Long non-coding RNAs

Non-coding RNA transcripts that are more than 200 nt long are termed long non-coding RNAs (lncRNAs). According to their location in relation to protein coding genes lncRNAs are divided into antisense, intronic, overlapping and intergenic lncRNAs. Their expression occurs in a stage- and tissue-specific manner making them good candidates to fine-tune the fate of different cells including T cells[103].

Genetic studies revealed lncRNAs such as *Xist* and *Airn*, to be involved in X inactivation and genomic imprinting, respectively, by regulating gene expression[104,105]. Different mechanisms have been described of how lncRNAs can interfere with gene expression. They can either (i) bind transcription factors, prohibiting them from binding to DNA, (ii) bind two or more proteins due to their tertiary structure, bring them into close proximity and guide them to DNA or (iii) result in chromosome looping similar to an enhancer like model[103].

1.3.5 Epigenetics and Multiple Sclerosis

More and more evidence has been given for epigenetic mechanisms being the bridge between genetics and environment in the pathogenesis of MS and explaining the 'hidden heritability' in disease inheritance.

Over the last years, accumulating evidence for parent-of-origin effects being involved in the etiology of MS has been provided. There is for instance a higher risk for maternal half-siblings to develop MS compared to paternal siblings, implying a parent-of-origin involvement in MS development[107]. Further evidence was given when it was shown by Chao *et al.* that the most prominent MS risk gene, *HLA-DRB1*15:01*, is more likely to be transmitted from mother-to-daughter compared to from father-to-daughter[108]. One of the best-characterized epigenetic manifestations that mediate parent-of-origin effects is the aforementioned genomic imprinting.

In addition to the observed parent-of-origin effects, different studies were conducted to detect DNA methylation changes in MS disease and further strengthened a role for epigenetics in MS disease etiology. All studies described in this paragraph performed genome wide methylation analysis in case-control cohorts utilizing the 450K methylation array.

In 2014, Graves *et al.* performed their analysis in $CD4^+$ T cells and detected differences in 38 regions with the most significant changes in the *HLA-DRB1* region. Interestingly, most of the differences detected in non-HLA regions mapped to genes that were previously associated with MS. In a follow-up study from the same group in $CD8^+$ T cells, a distinct set of 79 differentially methylated CpGs was detected[109,110].

Bos *et al.* investigated $CD4^+$ and $CD8^+$ T cells and whole blood and detected differences in global methylation levels in $CD8^+$ T cells but no genome-wide significant differences on a CpG level between cases and controls. This study further underlines the importance of purifying different cell types when performing genome-wide methylation analysis[111].

Another study performed by Huynh and colleagues uncovered subtle but significant and consistent changes when comparing normal appearing white matter between cases and controls. Several identified DMRs were related to oligodendrocyte genes or genes involved in oligodendrocyte survival[112].

Overall, the observed differences in methylation were rather subtle but consistent for all of these studies which stands in contrast to large differences often observed in cancer studies. Huynh *et al.* explained in their study this phenomenon with the complete disruption of the cell in cancer and provided evidence that these small changes were able to impact gene expression when they occurred in certain gene regions in other complex diseases. Recently, a complete review was released discussing subtle but consistent methylation changes as the hallmark of complex diseases[113].

A number of studies have also profiled miRNAs in MS patients[114], comparing different conditions, miRNA sources and using different platforms. After initial modest overlap between different studies, several miRNAs are now emerging as important regulators in MS. There is also emerging evidence for miRNAs termed NeurimmiRs that are implicated in both neuronal and immune processes mediating possibly the crosstalk between the two systems. The miRNAs miR-155 and miR-326 have been demonstrated to be dysregulated in PBMCs and $CD4^+$ T cells of MS patients and lead to an ameliorated EAE disease course when silenced. The same miRNAs were upregulated in active MS lesions in the brain[90].

2 AIMS

The low concordance rate of MS in monozygotic twins[26,28], the fact that only a part of disease inheritance and variance can be explained by all identified MS risk genes[18,29], the observed parent-of-origin effects[107,108] and changes in DNA methylation between cases and controls[114] all strongly suggest a role for epigenetic mechanisms in MS etiology, but the extent of their contribution and the underlying mechanisms are far from being understood.

In this thesis we set out to shed further light on how epigenetic mechanisms, in particular DNA methylation and ncRNAs, and their manifestations, such as genomic imprinting, impact the pathogenesis and inheritance of MS and its animal model.

We focus on the role of epigenetic mechanisms in regulating $CD4^+$ T cells functions due to the critical role of $CD4^+$ T cells in the etiology and pathogenesis of MS and EAE, and adaptive immunity in general.

3 METHODOLOGICAL CONSIDERATIONS

3.1 Epigenome-wide association studies in Multiple Sclerosis and its animal model

Epigenetic mechanisms in MS were investigated in this thesis by conducting different epigenome-wide association studies (EWAS). We carried out EWAS both in its classical sense, by investigating changes in DNA methylation, and we also investigated the consequences of DNA methylation in the form of genomic imprinting. We here set out to explore the role of the epigenome in inheritance and pathogenesis of EAE by utilizing a reciprocal backcross design to explore parent-of-origin effects, which can be caused by genomic imprinting. Further, a next generation sequencing (NGS)-based approach was used to survey genomic imprinting in $CD4^+$ T cells and to profile miRNAs at the initial stage of EAE development. With Illumina 450K, an array based technology, we studied genome-wide variation in DNA methylation in $CD4^+$ T cells and monocytes of MS patients and controls.

3.1.1 Using a reciprocal backcross design to identify parent-of-origin effects in MS-like disease

We studied the impact of parent-of-origin on genetic regulation of MS-like autoimmunity using a well-established model of MS under controlled breeding and environmental conditions. To identify parent-of-origin dependent loci that predispose for EAE on a genome-wide level, a reciprocal backcross between the EAE-susceptible DA and the MHC-identical EAE-resistant PVG rat strain was established (Fig.2). First an F1 generation was created by breeding DA females with PVG males (DAxPVG). Next we created two independent experimental populations, the DA backcross (DABC) population and the PVG backcross population (PVGBC) by breeding either DA rats with F1 hybrids or PVG rats with F1 hybrids in a reciprocal manner. In detail, DA females were bred with F1 males (DAxF1) and F1 females were bred with DA males (F1xDA) in the DABC population. In the PVG backcross population, we bred PVG females with F1 males (PVGxF1) and F1 females with PVG males (F1xPVG). This breeding regiment resulted in genetically unique animals enabling linkage analysis, which identifies genomic regions where a particular allele tends to be inherited together with the disease phenotype, while being able to track the parental origin of inherited alleles. For instance, in the DABC population, PVG alleles are either exclusively paternally (DAxF1) or maternally (F1xDA) inherited, whereas in the PVGBC population, it is the DA alleles that are either paternally (PVGxF1) or maternally (F1xPVG) inherited depending on the reciprocal cross. Using the two reciprocal backcross populations, DABC and PVGBC, enabled validation of identified parent-of-origin dependent disease loci.

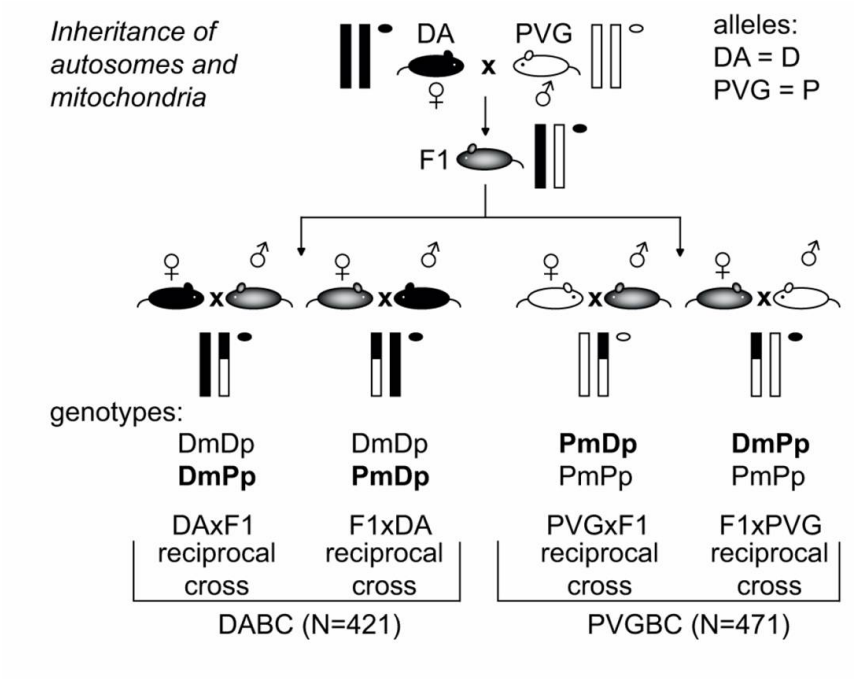

Figure 2: Schematic overview of reciprocal backcross breeding

3.1.2 Exploring the imprintome of CD4$^+$ T cells and tissues: an RNA sequencing based approach

The catalogue of imprinted genes in mice has been steadily increasing due to several studies that have been conducted in different tissues over the last years. With RNA sequencing becoming the method of choice to identify imprinted genes on a genome-wide level, several groups set out to quantitatively measure allele-specific expression in samples from reciprocal crosses of inbred mouse strains[56,57]. Allele-specific expression is quantified by counting reads with the reference allele as well as reads with the alternative allele using known single nucleotide variations (SNVs) to establish the parental origin of the allele. However, DeVaele *et al.* demonstrated in their validation study that this approach can also lead to a high rate of false positive findings due to an insufficiently robust statistical analysis, low number of biological replicates, mapping bias and a low library complexity[115].

In our study, we conducted RNA sequencing in rat reciprocal hybrids between the EAE-susceptible DA rat strain and the EAE-resistant PVG rat strain and tried to take most of these considerations into account to provide a robust survey of imprinted genes in a so far unexplored data set of sorted CD4$^+$ T cells and tissues in the rat. We employed large number of independent biological replicates (17 individuals per cross) and a starting amount of 1-2 µg of input RNA that should reduce low library complexity issues. We considered only independent SNVs (separated by a distance greater than the read length of 100 bp) that showed sufficient coverage of 24X in the initial genome-wide discovery and 10X when multiple consecutive SNVs where further investigated. Calling of imprinted genes was based on the true false discovery rate of 4 % ($p < 10^{-7}$), the degree of allele specific bias with a previous identified cut-off of 65% and the validation using targeted sequencing in independent samples. Since recurrence of imprinting across related tissues has been reported,

we performed RNA sequencing in two immune tissues which enabled us to get further evidence for imprinted candidate genes identified in the $CD4^+$ T cells. Additionally, unlike all other studies that sequenced poly-A containing RNAs, we sequenced all RNA species depleted of ribosomal RNA with retained strand information that allowed investigation of non-coding and/or antisense transcripts.

3.1.3 Classical epigenome-wide association studies in Multiple Sclerosis: a 450K DNA methylation approach in $CD4^+$ T cells and monocytes

Both of our methylation studies in MS were, as most of the EWAS studies up to date, conducted in a case-control cohort comprising individuals that are collected based on their phenotype *i.e.* MS cases at different stage of disease and healthy controls. Considering the history of disease with onset in adults and long disease duration, this is the most feasible type of a cohort to study. To detect methylation differences between cases and controls in $CD4^+$ T cells and monocytes, we utilized widely-used Illumina 450K beadchip arrays, which detect 485.000 CpG sites throughout the genome. Considering available DNA amount from sorted cells, costs and throughput, 450K arrays were the most practical way to detect methylation genome-wide. Moreover, as 450K arrays are widely used by other investigators, this enables comparison with other studies and gives a possibility to replicate our findings. For instance, we investigated our findings in the *MIR21* locus in $CD4^+$ T cells in two other MS case-control cohorts from Norway and Australia. Having this data available improved the interpretability of our findings and enabled us to strengthen our hypotheses.

3.2 VALIDATION APPROACHES

Different technical and functional validation approaches were applied in this thesis.

RNA sequencing approaches suffer from a high rate of false positive calls due to extensive data processing and statistical analysis, which makes validation in an independent sample cohort and with an independent method almost indispensable. We chose to validate our findings in an independent smaller sample cohort comprising three individuals per cross as biological replicates and one technical replicate to confirm known and potential novel imprinted genes. Out of selected candidate imprinted genes, 17 were validated giving a total validation rate of 77.3% (17/22). Additionally, we obtained further evidence by investigating several tissues, some functionally related, from the same individuals.

The use of a transgenic mouse model expressing elevated levels of the imprinted *Dlk1* gene enabled us to carry out a functional validation and confirm *Dlk1* as a potential candidate gene mediating observed parent-of-origin effect in EAE. The functional importance of *Dlk1* was shown by its involvement in the underlying immune responses, which also gave further insights into mechanisms of its function in disease.

Since increased technical processing and complicated analysis pipelines can also be an issue in the analysis of Illumina 450K methylation data, we applied technical pyrosequencing validation of representative CpGs in both of our studies in MS. However, power is always an

issue in studies of complex and heterogeneous diseases such as MS. Therefore, we attempted to replicate our findings in independent cohorts. Additionally, when such cohorts or data types were not readily available, we tried to gain further support from analyzing additional omics data such as genome and transcriptome. This approach can provide additional functional interpretations of detected differences.

Nevertheless, unlike genetic studies, there is always an issue of reverse causality in EWAS *i.e.* is the identified epigenetic change causing the phenotype or is it a consequence of the phenotype. We attempted to address this issue by investigating DNA methylation changes as a potential mediator of the genetic variation that causes disease. In addition, we utilized an *invitro* reporter system to confirm that a change in DNA methylation at a given locus has a potential to impact transcription of the locus.

4 RESULTS

In the studies included in this thesis we set out to investigate if and how epigenetic mechanisms contribute to inheritance and pathogenesis of MS and its animal model EAE.

4.1 Parent-of-origin affects susceptibility to EAE

Several studies implicate epigenetic mechanisms in the inheritance of MS. For example, parent-of-origin effects have been detected in multigenerational MS studies[107,108]. The best-characterized epigenetic mechanism that causes parent-of-origin effects is genomic imprinting which itself is regulated by one of the most-studied epigenetic mechanisms i.e. DNA methylation[58,59,60,61,62]. Thus, genomic imprinting may provide one explanation for the observed effects in MS. However, parent-of-origin effects are challenging to study in human population as large multigenerational cohorts are rarely available and potential environmental cofounders are difficult to account for.

Therefore, in **Study I** we addressed the impact of parent-of-origin using a well-established model of MS that closely mimics human disease, and two controlled large reciprocal backcrosses between the strains with well-characterized genetic regulation of disease. We uncovered that 37% (6/16) and 54% (6/11) of all loci that were identified to predispose for EAE in the reciprocal backcross with the susceptible and resistant strain, respectively, depend on the parental origin of the disease predisposing allele. Several mechanisms may explain parent-of-origin effects; however, apart from the influence of the Y chromosome, we did not observe strong evidence for other genetic mechanisms. In contrast, the majority of parent-of-origin dependent loci displayed effects resembling genomic imprinting suggesting involvement of epigenetic mechanisms. Of these, several overlapped well-known clusters of imprinted genes *i.e*. *Gnas*, *Igf2r* and *Dlk1-Dio3*, which contain members known to control immune functions and may mediate the effect of detected loci. For example, a locus on rat chromosome 6 was only associated with EAE when the disease-predisposing allele was paternally inherited, resembling an imprinting-like pattern (Fig.3). Interestingly, this locus overlaps a well-known imprinted cluster *i.e.* the *Dlk1-Dio3* cluster. The fact that only *Dlk1* from the genes tested in the locus displayed a lower expression when the disease-predisposing allele was paternally inherited and that it had been associated with autoimmune diseases before made it a good candidate gene to mediate the observed parent-of-origin effect on chromosome 6.

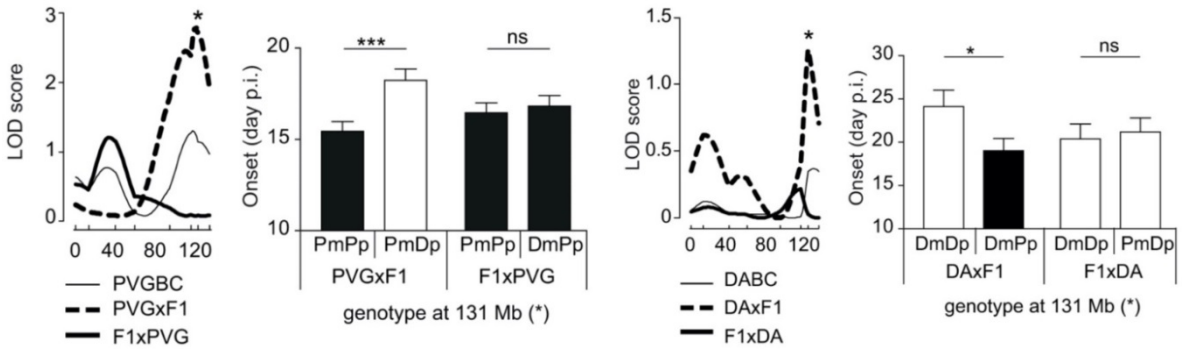

Figure 3: Detection of a parent-of-origin dependent EAE risk locus on rat chromosome 6 when the disease-predisposing alleles were paternally inherited

Taking into account parental origin enabled us to identify multiple novel loci that predispose for disease, while the majority of loci that did not depend on the parental origin has been previously reported. This study highlights the involvement of epigenetic mechanisms in the inheritance of MS-like disease and underlines how the incorporation of parent-of-origin into conventional studies can potentially lead to the identification of novel risk genes like the imprinted *Dlk1* gene.

4.2 Imprinted genes affect susceptibility to develop EAE and may control T cell function

Beside their role in development, imprinted genes have become more and more implicated in the regulation of immune responses in inflammatory diseases. Due to our observations in **Study I** that the paternally inherited disease-predisposing allele in the *Dlk1-Dio3* locus also predisposes for lower *Dlk1* expression in the backcross rats, we speculated that paternally expressed *Dlk1* gene may control susceptibility to EAE. Experiments conducted in trangenic mice expressing double dosage of *Dlk1* compared to their wild type littermates revealed that reduced levels of *Dlk1* drive more severe disease by modulating the T and B cell response in EAE and support our hypothesis that *Dlk1* is at least partially responsible for the previous observed parent-of-origin effects on chromosome 6 (Fig.4).

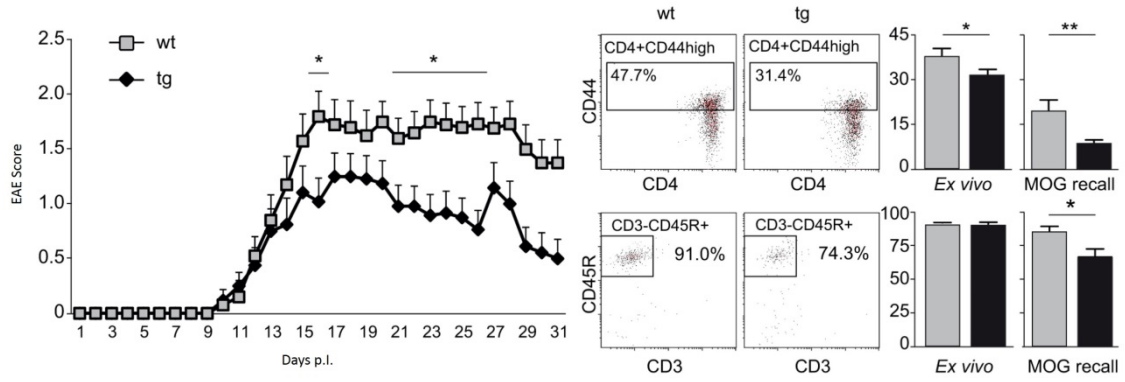

Figure 4: Ameliorated EAE disease course and modulated immune response, *ex vivo* and after restimulation with MOG antigen, in *Dlk1* transgenic mice expressing elevated levels of *Dlk1*.

To identify additional imprinted genes that could potentially mediate the susceptibility to EAE, we conducted in **Study II** a genome-wide identification of genes that express parental allelic bias in $CD4^+$ T cells, thymus, spleen and brain. By using an RNA sequencing based approach in reciprocal F1 hybrids we were able to identify and validate seven imprinted autosomal loci, six of them being well-known imprinted loci (*Igf2r*, *Peg12*, *Kcnq1*, *Gnas*, *Zrsr1* and *Impact*) and one novel locus overlapping the cluster of C-type lectin receptors in $CD4^+$ T cells (Fig.5). Imprinted genes in the C-type lectin receptors, *Igf2r* and *Gnas* clusters might mediate effects of the parent-of-origin EAE QTLs on chromosome 4, 1 and 3, respectively, identified in **Study I**.

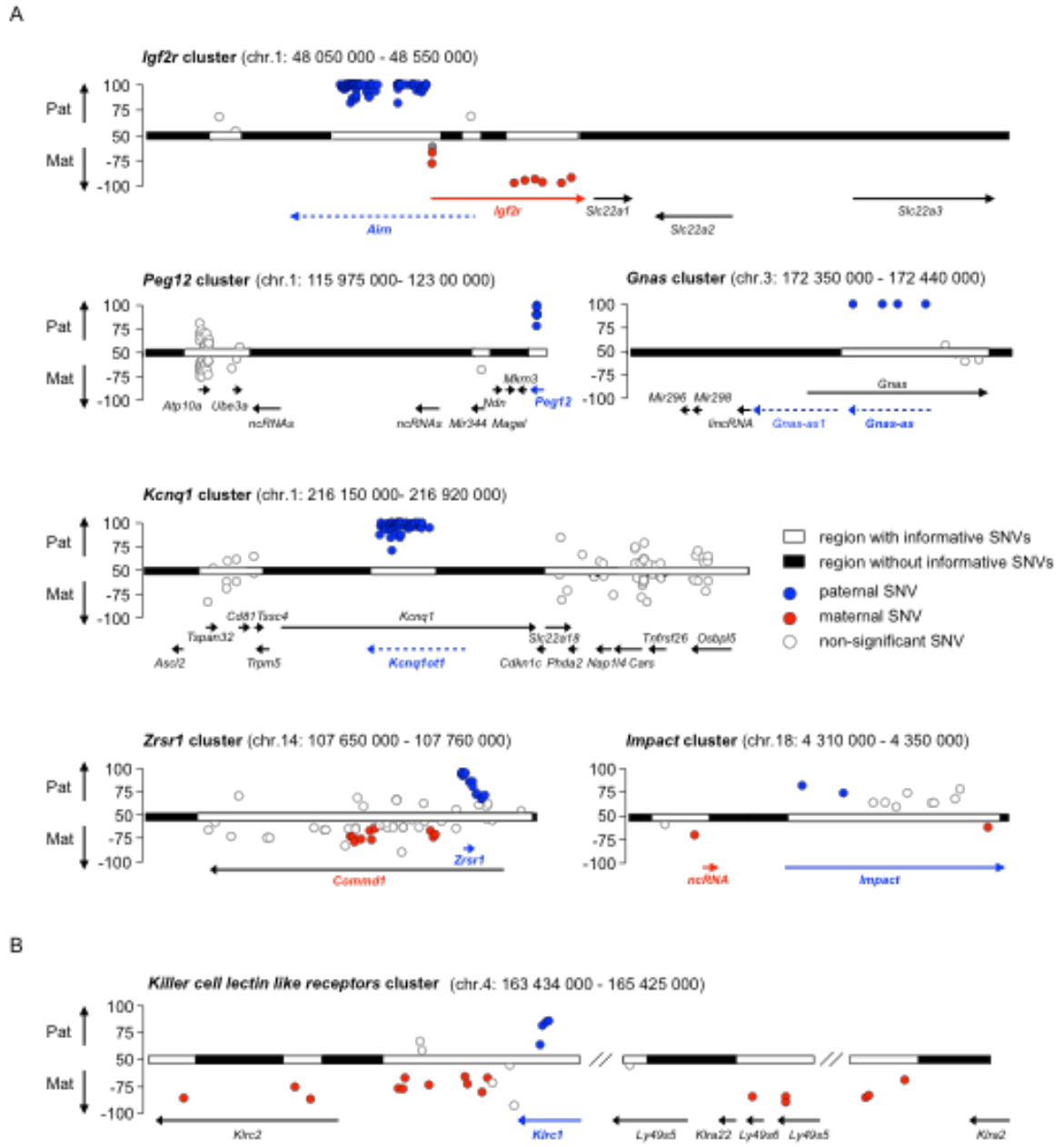

Figure 5: Identified known (A) and potential novel (B) imprinted loci on autosomes in $CD4^+$ T cells from adult rat reciprocal hybrids.

We further observed that most of the imprinted genes located on autosomes including not yet annotated lncRNAs were preferentially paternally expressed. Those imprinted lncRNAs were detected in the well-known imprinted clusters of *Igf2r* and *Kcnq1* on chromosome 1 and in the *Gnas* cluster on chromosome 3 likely representing lncRNAs *Airn*, *Kcnq1ot1* and *Gnas-as1* (*Nespas*), known to associate with these clusters in other species[116,117,118]. In contrast to imprinted genes on the autosomes, imprinted transcripts on the X chromosome displayed preferential expression from the maternal allele, which we identified to be regulated by the parent-of-origin and genetic background in adult rats.

While studying imprinting across several tissues and developmental stages, we demonstrated that (i) imprinting is tissue-specific and associates with tissue-specific lncRNAs, (ii) parental expression of imprinted genes may differ between the tissues and, most interestingly, (iii) there are novel candidate imprinted genes with well-known immune functions that are imprinted only in the brain and display a shift between parental alleles during early life.

Our data from **Study I and II** demonstrate how imprinted *Dlk1* interferes with the underlying immune response in MS-like disease by mediating changes in T and B cell activation and provide further insights into the underlying mechanisms on how parent-of-origin might impact the immune response by affecting $CD4^+$ T cell function through genomic imprinting, lncRNAs and X inactivation skewing. At the same time we extend the catalogue of imprinted genes by a survey of imprinted genes in $CD4^+$ T cells, thymus, spleen and brain in the rat.

4.3 Non-coding RNAs as mediators of epigenetic mechanisms in EAE and MS

The vast majority (90%) of the imprinted autosomal SNVs identified in $CD4^+$ T cells in **Study II** that displayed strong paternal bias (96% expression from the paternal allele on average) belong to lncRNAs. Those imprinted lncRNAs have been associated with the regulation of genomic imprinting itself in *cis*[116,117,118] but accumulating evidence also suggests a *trans* acting role for the imprinted lncRNAs[119,120].

Besides lncRNAs, clusters of small non-coding RNAs, including miRNAs, are also known to reside in imprinted loci. The largest cluster of imprinted miRNAs (including miR-127, miR-434, miR-136, miR-379, miR-134, miR-541, miR-369) is located in the aforementioned *Dlk1-Dio3* cluster (Fig.6) that we identified to be overlapping with a parent-of-origin EAE QTL on rat chromosome 6 in **Study I**. Indeed, we detected all of the imprinted miRNAs in this cluster to display higher expression in the susceptible strain during induction of EAE in **Study III**.

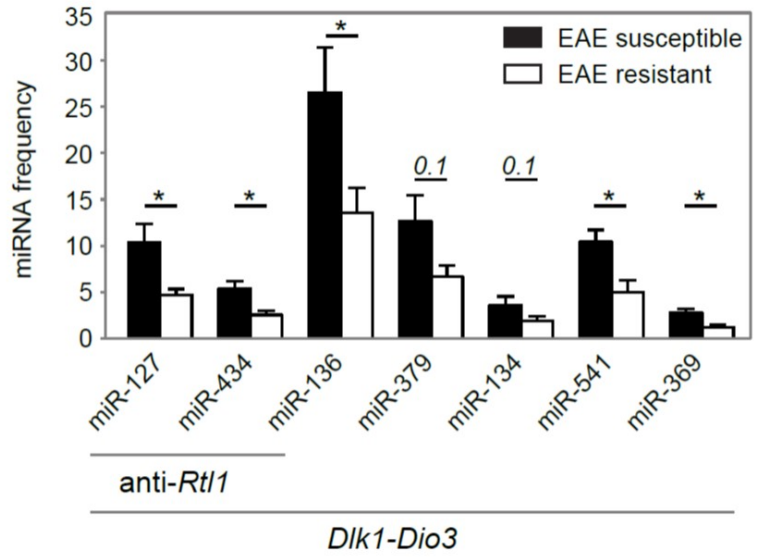

Figure 6: Expression of imprinted miRNAs in the well-known imprinted *Dlk1-Dio3* cluster.

In **Study III** we set out to characterize miRNAs that are involved in the early immune response in rats differing in their susceptibility to EAE. Overall we identified 544 miRNAs in the draining lymph nodes after EAE induction. Out of 329 miRNAs that could be reliably quantified, 43 miRNAs were differentially expressed between the strains. Most of the differentially expressed miRNAs (35) displayed higher expression in the susceptible strain whereas only eight miRNAs were higher expressed in the resistant strain. Only 1/3 of tested differentially expressed miRNAs showed also differential expression in naïve lymph nodes indicating that most of the regulated miRNAs are EAE dependent. Target genes of higher expressed miRNAs in both strains identified by using computational prediction tools and whole genome expression data revealed an involvement in functions that are important for MS and EAE like immune cell migration (Cxcr3) and cellular maintenance and signaling (Prkcd, Stat1). By far the most abundant was miR-21, a miRNA that has already been associated with autoimmune diseases[121,122,123,124], and that showed higher expression in the susceptible DA strain during the initial stage of EAE.

Interestingly, when investigating methylation in $CD4^+$ T cells from MS patients in **Study IV** on a genome-wide level, we identified, among the most significant hits, multiple CpG probes that map to the last two exons of the *VMP1* and the entire *MIR21* gene on chromosome 17. Here, we observed a significantly higher methylation at all eleven consecutive CpG sites in RR-MS patients in relapse when compared to SP-MS patients and healthy controls (Fig.7). At the same time we demonstrated that the increased methylation levels associated with lower expression of mature miR-21, but not *VMP1*, in RR-MS patients supporting a functional impact of methylation levels on the expression of miR-21. Enrichment analysis for miR-21 target genes, identified using RNA sequencing in $CD4^+$ T cells, revealed that there was a significant overrepresentation of miR-21 target genes among the up-regulated genes in $CD4^+$ T cells of RR-MS patients.

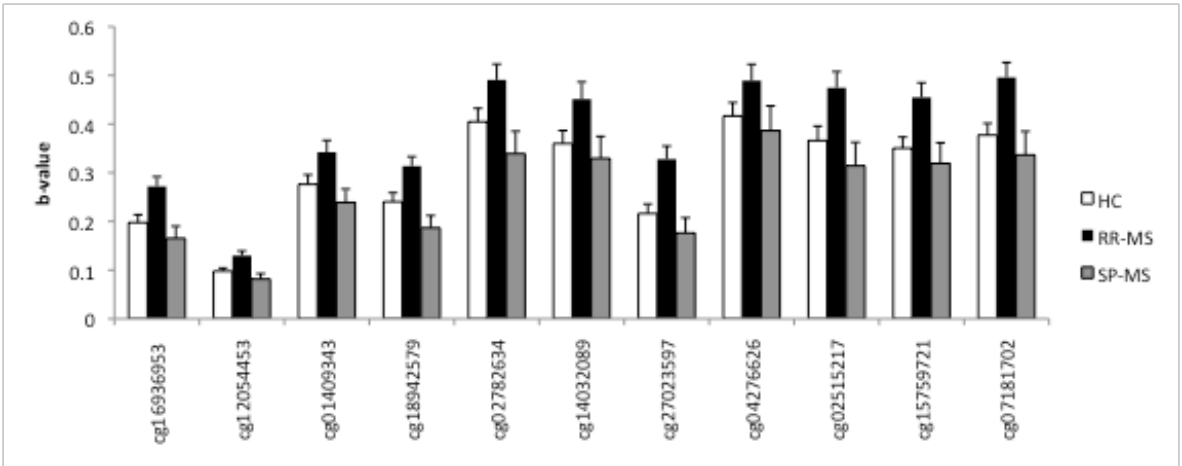

Figure 7. Hypermethylation at *MIR-21* locus at all 11 consecutive CpGs on human chromosome 17.

Overall, **Study II, III and IV**, strongly support involvement of ncRNAs, lncRNAs and miRNAs, in regulating immune responses occurring in EAE and MS and suggests another way of how epigenetic mechanisms and genomic imprinting can affect the immune responses through the regulation of miRNAs and their target genes.

4.4 DNA methylation as a mediator of risk factors in MS disease

In **Study IV** we described how the disease state impacts DNA methylation in $CD4^+$ T cells at a specific locus, *MIR21*, and how this can affect the underlying immune response in MS. Using a similar approach we studied DNA methylation changes in monocytes from MS patients in **Study V**. Here we identified significant methylation changes at multiple CpG sites that mapped to the *HLA-DRB1* gene on chromosome 6 with MS patients displaying lower methylation levels when compared to controls. Knowing that the primary HLA effect in MS is mediated by the *HLA-DRB1*15:01* allele, which is the strongest genetic risk factor in MS, we performed analysis in carriers and non-carriers and observed significantly lower methylation levels for homozygous carriers when compared to heterozygous carriers or non-carriers (Fig.8). We further demonstrated allele-specific hypomethylation of *HLA-DRB1*15:01* that associated with higher expression of the allele and contributed to an overall higher expression of *HLA-DRB1* in the carriers. The identified differentially methylated region in *HLA-DRB1* displayed methylation-sensitive promoter and enhancer capabilities in an *invitro* reporter system.

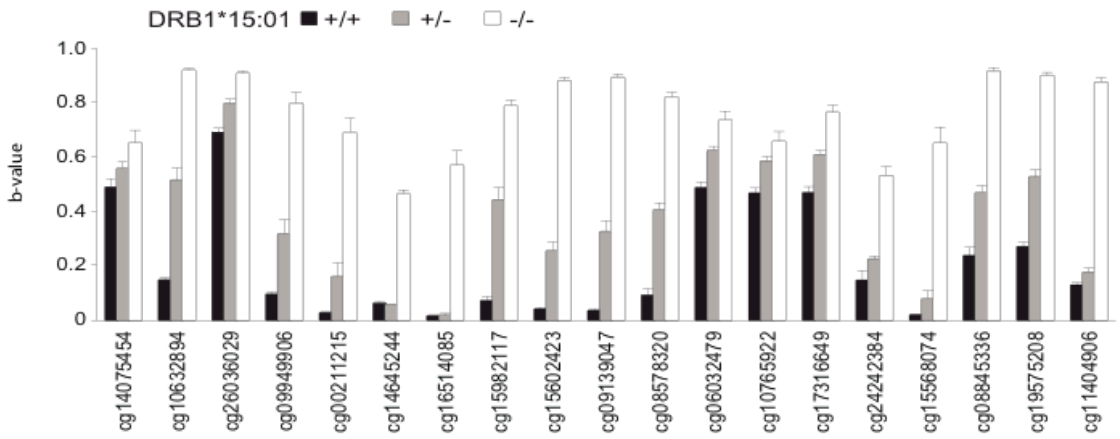

Figure 8: *HLA-DR15:01*-dependent hypomethylation at the *HLA-DRB1* gene on human chromosome 6.

We then set out to address a role for DNA methylation as a mediator of genetic risk in MS genome-wide by performing Causal Inference Test (CIT) in a cohort of MS cases (n=140) and healthy controls (n=139). We identified 50 single nulcleotide polymorphisms (SNPs), all of them being located in the extended HLA region, whose association with the disease was dependent on DNA methylation changes at seven DMRs, four of which overlapped DMR detected in *HLA-DRB1* in monocytes. Most of them were shown to mediate disease risk of the *HLA-DRB1*15:01* allele using conditional association analysis in a large cohort of MS cases (n=8172) and controls (n=13263). Interestingly, three SNPs still conferred disease risk after adjusting for all known MS associated alleles in the HLA locus representing potential novel risk gene(s). These data strongly support our hypothesis that DNA methylation mediates, in part, the effect from the *HLA-DRB1*15:01*, and potentially other gene(s) in the locus and ascribes a causal role of DNA methylation in MS development. Unlike the HLA, we could not demonstrate in **Study IV** a mediator role for DNA methylation for MS risk in the aforementioned *MIR21* locus in $CD4^+$ T cells under tested conditions.

This study highlights the role of DNA methylation as a mediator of genetic risk in MS disease and provides further insights into how methylation interferes with the underlying immune responses in MS through the higher expression of the *HLA-DRB1* gene encoding the HLA class II molecules that present antigen to $CD4^+$ T cells.

5 DISCUSSION AND FUTURE DIRECTIONS

Over the last years a growing body of evidence suggests involvement of epigenetic mechanisms in the pathogenesis of complex diseases and our data contribute such evidence in Multiple Sclerosis.

5.1 Epigenetic mechanisms of imprinted genes modulate pathogenesis of complex inflammatory diseases

We demonstrate that more than 30% of genomic loci that predispose for EAE between the DA and PVG rat strains depend on the parental origin of the disease-predisposing allele. This implicates significant parent-of-origin effects in the animal model of MS supporting previously suggested effects in MS[107,108]. A pattern of identified parent-of-origin effects further suggests that epigenetic mechanisms controlling genomic imprinting modulate pathogenesis of EAE.

Taking into account parental origin led to identification of novel risk loci, several of which overlap well-known clusters of imprinted genes, increasing our knowledge of immunopathogenic mechanisms in EAE. The exclusively maternally expressed *Igf2r* gene, which we showed to be maternally expressed in $CD4^+$ T cells in rats after EAE induction, has previously been shown to be involved in T cell activation[125] and could potentially mediate the observed effect of the locus on chromosome 1. We provide further evidence supporting the role of paternally expressed *Dlk1* gene in mediating effects of the locus on chromosome 6. In inbred and backcross rats, we demonstrate that the paternally inherited risk allele predisposes for lower *Dlk1* expression. In transgenic mice, we confirmed that lower *Dlk1* expression leads to more severe disease and modified T and B cell responses. Dlk1 is known to inhibit Notch signaling by acting as a Notch antagonist[126]. In line with our results, previous studies have shown that the inhibition of Notch signaling can lead to no or less severe EAE in mice[127]. Further, increased Notch signaling has been shown to enhance T cell proliferation[128] and to prevent activated T cells from entering apoptosis[129]. Additionally, we provide evidence for a novel cluster of imprinted genes in $CD4^+$ T cells, comprising C-type lectin receptors, which might mediate the effect of the locus on chromosome 4. We validated paternally expressed *Klrc1*, which is in proximity of the previously reported imprinted *Klrb1f*[130] gene, further strengthening its imprinting status since novel imprinted genes tend to appear in regions that have already been identified as imprinted and may rely on already existing mechanisms of regulation. In addition to *Klrc1*, there are potentially several other C-type lectin receptors in the locus *e.g.* maternally expressed *Klrc2*, which were also detected in immune tissues from adult rats. C-type lectin receptors are expressed predominantly on the surface of NK cells but also $CD4^+$ T cells and have been shown to regulate adaptive immune responses[131,132,133] and potentially cause direct damage in MS[134].

All together our data suggest that imprinted genes may modulate immune responses relevant for development of EAE and MS, in particular function of $CD4^+$ T cells. What is the exact role of genomic imprinting, which is restricted to a small set of genes and has its main functions in early development, in controlling the immune system and susceptibility to inflammatory diseases, remains to be established. A loss of imprinting at the *IGF2* locus, leading to an increased expression of *IGF2*, has been observed *invitro* after stimulation of naïve T cells[79]. In Rheumatoid Arthritis patients, loss of *IGF2* imprinting relates to the degree of inflammation and leads to an enhanced cell growth and proliferation mediated by increased *IGF2* expression in synovial fluid cells[135]. Both observations suggest that an inflammatory environment, which also exists in the lymph nodes and CNS in EAE and MS, can influence the imprinting status and expression of imprinted genes. Furthermore, Wang *et al.* demonstrated biallelic expression of genes related to the immune system located in one of the biggest clusters of imprinted genes, the X chromosome, in T cells from patients with systemic lupus erythematosus[136]. It is tempting to speculate that inflammatory stimuli, as well as other factors, may interfere with proper epigenetic regulation of imprinting and increase expression of imprinted genes which in turn may lead to increased cellular growth and proliferation contributing to chronic inflammation. Additionally, the intact imprinting status will dictate what alleles are being expressed, which may lead to alternative outcomes when the risk alleles are considered. The existence of imprinted gene networks (IGNs) comprising not only imprinted genes but also biallelic genes further adds to the complexity of how imprinted genes can impact immune responses. The IGN investigated by Al-Adhami and colleagues was identified to be involved in the control of the cellular composition of the extra cellular matrix, which impacts both T cell migration in inflamed tissue[137] and differentiation of oligodendrocyte precursor cells into mature oligodendrocytes (unpublished data Bachelor thesis), both important functions involved in the pathogenesis of EAE and MS.

Taken together, our data highlight the importance of incorporating parent-of-origin effects and allelic expression bias in conventional genetic and genomic studies in the future. This might address, at least in part, the issue of 'hidden heritability' and lead to the identification of novel MS risk genes. It may also facilitate characterization of the molecular mechanisms of such risk genes increasing our knowledge of pathogenic mechanisms.

Imprinted miRNAs also reside in well-known imprinted gene clusters and these miRNAs have a potential to regulate genes related to MS and other inflammatory diseases. The largest cluster of imprinted maternally expressed miRNAs is located in the aforementioned *Dlk1-Dio3* cluster that we identified to overlap the parent-of-origin EAE locus on rat chromosome 6. Several miRNAs from this cluster have been predicted to target the major MS risk gene, *HLA-DRB1*[138]. This may provide a mechanistic explanation for previously reported parent-of-origin effects mapping to the *HLA-DRB1* locus in MS[108]. However, no miRNAs have been experimentally shown yet to target *HLA-DRB1* and this hypothesis needs further investigation. Additionally, pathway analysis performed on predicted targets of miRNAs located in the imprinted *Dlk1-Dio3* locus reveal functions like T cell proliferation and T cell

activation, both crucial processes during the early immune response in EAE and MS[138]. We found that several of these miRNAs show higher expression in the EAE-susceptible strain compared to the EAE-resistant strain during early stages of EAE induction. This suggests a role for imprinted miRNAs, besides *Dlk1*, in mediating the observed parent-of-origin effect in the locus. Changes in miRNA expression due to loss of imprinting at ICRs have been implicated in complex diseases[139].

Interestingly, the majority of autosomal imprinted transcripts in $CD4^+$ T cells exerted strong paternal expression bias. Among them we uncovered previously not annotated paternally expressed antisense lncRNAs, *Airn*, *Kcnq1ot1* and *Gnas-as1* in the rat located in the *Igf2r*, *Kcnq1* and *Gnas* cluster, respectively. Besides their role in regulating gene expression in *cis*[116,117], accumulating evidence suggests that lncRNAs can exert their repressive function also in *trans* forming aforementioned functional IGNs in different tissues and cells. For instance, *H19*, a lncRNA in the *Igf2/H19* locus, has been shown to control embryonic growth through regulation of IGN of imprinted genes in *trans*[119]. It is tempting to speculate that paternal lncRNAs in $CD4^+$ T cells may engage in interaction with a large number of genes in *trans* to control T cell functions. This is further supported by the observation in reciprocal F1 hybrids showing that paternally transmitted susceptible alleles confer activation of several signaling pathways resulting in higher proliferation of $CD4^+$ T cells.

Very little is still known about the action of lncRNAs. To investigate potential targets of the imprinted lncRNAs in *trans* and how they impact T cell function, so called guilt by association studies need to be performed. The bioinformatics methods allow for the identification of lncRNAs and protein-coding genes that are tightly co-regulated[140]. In that way known functions of the protein-coding genes provide hints for the functions of the lncRNA of interest. However, to fully investigate the function of a lncRNA, gain and loss of function studies need to be performed similar to studies by Stelzer *et al.* done on the *IPW* lncRNA[120].

Investigation of different classes of ncRNAs may reveal novel mechanisms of the control of immune responses and inflammatory diseases. This can provide in the future basis for novel interventions targeting cell type specific networks of genes using RNA-based therapeutics[141].

5.2 Epigenetic mechanisms control immune reactions in MS

To get further insights into immunopathogenic processes in MS we studied DNA methylation, which can actively impact gene regulation on the transcriptional level or be a marker of the genome activity[142], in $CD4^+$ T cells and monocytes from MS patients.

In $CD4^+$ T cells from MS patients we detected no genome-wide significant changes in DNA methylation. However, we observed subtle but significantly higher methylation changes at eleven consecutive CpG sites covering a locus encoding the *MIR21* gene in RR-MS patients in remission compared to SP-MS patients and healthy controls. It is important to keep in

mind that the bulk of the $CD4^+$ T cell population comprising different $CD4^+$ T cell subsets was investigated. This could be a reason for the lack of genome-wide significant changes as potentially only a minor fraction of 'pathogenic' T cells might carry epigenetic differences. In addition, observed results could just represent differences in the cellular composition of the $CD4^+$ T cell subsets between patients and controls. However, considering that it has been shown that *MIR21* is hypomethylated in Th1/Th2[143] and Tregs[144] compared to naïve T cells and that miR-21 expression is the lowest in naïve $CD4^+$ T cells compared to other $CD4^+$ subsets[145], the observed hypermethylation of *MIR21* is less likely caused by differences in cell frequencies. Moreover, increased methylation levels at the *MIR21* locus strongly associated with lower expression of mature miR-21 in RR-MS patients implying a functional impact of DNA methylation on the expression of miR-21. Additionally, enrichment analysis for miR-21 target genes revealed that there was a significant overrepresentation of miR-21 target genes among the up-regulated genes in $CD4^+$ T cells of RR-MS patients, irrespective of the target prediction tool or enrichment analysis method. Most of the mRNA targets of miR-21 in $CD4^+$ T cells have been shown to be involved in processes with a possible anti-apoptotic and pro-proliferative effect, which stands in contrast to the rather pro-apoptotic role of miR-21 observed in cancer and other autoimmune diseases[146,122,123,124]. However, in most of these studies investigations were focused on a single mRNA target of miR-21, whereas it is well documented that a single miRNA tends to target multiple, often functionally related genes.

Elevated levels of miR-21 in heterogeneous tissues represent a sign of inflammation but the exact roles of miR-21 in different immune cells and conditions are still under investigation. This is similar to findings from EAE by us and others[147,148] showing up-regulation of miR-21 during EAE induction and a significantly higher expression in the EAE-susceptible strain. In MS, miR-21 expression has also been reported to be up-regulated during the relapse phase in PBMCs from RR-MS patients when compared to SP-MS patients and controls[149]. In line with these findings, deletion of *MIR21* in mice leads to protection from EAE[121]. This is in contrast to our observations of hypermethylation and lower expression of miR-21 in $CD4^+$ T cells of RR-MS. Similar to our findings in RR-MS in remission, miR-21 expression was reported to be down-regulated in PBMCs during remission in RR-MS patients compared to controls[150]. Therefore, miR-21 seems to play different roles at different stages of disease depending for instance on the specific $CD4^+$ T cell subset and the availability of its target genes. In regard to T cells a different expression pattern for miR-21 has even been observed for different $CD4^+$ T cell subsets[145]. To fully investigate the function of this highly abundant miRNA well-powered cohorts and pure cell subtypes will be needed, accompanied by functional investigations using for example inducible conditional *MIR21* deletion models in mice.

In monocytes from MS patients, we uncovered the most significant changes, encompassing a large number of CpG sites, in the HLA locus and specifically in the region encompassing exon 2 and surrounding intronic sequences of the *HLA-DRB1* gene. The *HLA-DRB1* locus has been denoted as the strongest genetic association to MS for more than 40 years now. Accordingly, we found that the major risk haplotype *i.e.* the *HLA-DRB1*15:01* is

hypomethylated and predominantly expressed compared to several other tested haplotypes, contributing to overall higher expression levels of *HLA-DRB1* in the carriers of the risk haplotype. These observations strongly suggested that, together with the structural characteristics of the peptide binding groove, *HLA-DRB1* expression levels are most likely the so far missing co-mediator of the *HLA-DRB1*15:01* risk haplotype. Indeed, recent studies have pointed to a relationship between *HLA-DRB1*15:01* and the levels of *HLA-DRB1* expression[151,152,153]. Our data further suggest that this effect is mediated though DNA methylation. To support this hypothesis, we demonstrated that there is a strong negative correlation between DNA methylation in the locus and expression of *HLA-DRB1* in monocytes, and that 5-aza treatment of PBMCs leads to higher expression of *HLA-DRB1*. Additionally, using an *invitro* reporter system, we demonstrated that the identified locus can act in a methylation-sensitive manner as an enhancer or a promoter. In line with our findings other recent studies investigating the interaction between the genome and epigenome in immune diseases and food allergy have found association with genetic variants and DNA methylation mapping to multiple loci in the HLA class II region[154,155,156]. To further strengthen the causal role of DNA methylation in causing disease, we performed CIT in a case-control cohort, followed by association analysis conditioning on the known MS risk variants. The majority of significant SNPs identified by CIT conferred the risk from *HLA-DRB1*15:01*, strongly supporting DNA methylation as a mediator of the risk. Interestingly, five SNPs still showed evidence of association after adjusting for all known MS associated variants suggesting novel, methylation-dependent, associations in the HLA locus. None of these SNPs has been previously independently associated with the risk of developing MS and they require replication in independent materials.

Our data in monocytes strongly suggest that genetic variation predisposes for different DNA methylation levels in the *HLA-DRB1* locus. However, we could not establish what confers differences in DNA methylation in the *MIR21* locus in $CD4^+$ T cells. We attempted to test the influence of genetic variation in this locus, also known to associate with MS[29] but found no evidence under tested conditions. Smoking, a well-known environmental factor in MS[35], also known to induce changes in DNA methylation[42], had no effect on either *HLA-DRB1* or *MIR21* methylation. We speculate that inflammation itself may trigger DNA methylation in MIR21, based on the observed hypermethylation in RR-MS and inflammatory neurological disease controls as well as association with inflammatory markers. Inflammation-induced methylation may form a feed-back loop in miR-21 regulation, which is a common mechanism of action of miRNAs, and in this case additionally controlled by epigenetic mechanisms. That this locus is prone to epigenetic regulation can be observed in several other diseases that report methylation changes.

Our data demonstrate how DNA methylation can act as a co-mediator of MS risk gene by regulating the expression of HLA class II molecules in APCs, which in turn may modulate activation of $CD4^+$ T cells. Additional epigenetic changes in $CD4^+$ T cells, potentially triggered by inflammation itself, may further modulate their function in disease. These studies also exemplify how the integration of genome and epigenome data can lead to the

identification of so far not appreciated risk variants and form a basis for their further functional investigations. Importantly, identification of epigenetic changes that cause inflammatory disease or contribute to disease progression may open up for future therapeutic interventions based on targeting disease epigenome.

5.3 Important considerations

Considering our own experience and EWAS performed by others in the field, the following general aspects should be taken into account to increase the interpretability of future epigenetic studies in MS and other diseases with similar complex etiologies.

A discovery cohort with appropriate size should be selected that provides sufficient power to test the hypothesis of interest. Clinical and lifestyle information for participants should be acquired to correlate epigenetic data with clinical parameters, treatment status and potential environmental risk factors but also to allow for the selection of a replication cohort that is similar to the discovery cohort.

The cell type of interest should be purified to exclude that observed changes in an epigenetic mark occur due to differences in frequency of cell subsets or that prominent changes in a specific cell subset disappear in the bulk of the overall cell population. With even single cells displaying a different methylome epigenetic studies conducted on a single-cell level should be considered in the future.

Analyses of epigenome-wide studies should be conducted using standardized pipelines to allow for data replication in independent sample cohorts from different laboratories.

Methods that provide better genome-wide coverage should be considered. Ideally, full genome studies should be performed using for example whole genome bisulfite sequencing. This is especially relevant as we still do not know where in the genome cell type- or disease-relevant epigenetic changes occur. This might be, however, difficult in large cohorts, primarily due to high costs and intensive analysis load, but feasible and better alternatives to 450K are emerging. For example, lllumina has recently released the EPIC bead chip, which comprises 90% of 450K probes with additional coverage of enhancer regions.

Additional epigenetic marks should be considered in future studies as DNA methylation represents only one of the mechanisms of epigenetic regulation. In that respect, it is also important to consider that the most widely used approaches to study DNA methylation based on bisulfite treatment do not distinguish between 5-methylcytosine and 5-hydroxymethylcytosine, which might have different functional impact on gene regulation[158].

Addition of further omics data, especially genome and transcriptome, should accompany epigenome studies to allow for a better interpretability of observed changes in epigenetic marks, including novel statistical and bioinformatics tools to integrate multi-omics data.

Finally, it is extremely relevant to establish the causality of identified epigenetic changes. With the recent advent of epigenome-targeting using for instance CRISPR/dCas9 fused with

catalytic domains of epigenetic modifiers[159], it might be possible in the future to directly assess the impact of an epigenetic change in a relevant cell type.

Collectively, I hope that my work has provided a better understanding of how epigenetic mechanisms contribute to the pathogenesis of MS, and complex diseases in general, and further insights into factors that need to be considered in future studies.

6 ACKNOWLEDGEMENTS

Without all of you this have never have happened! That is why I would like to take the opportunity to say THANK YOU to

My main supervisor Dr. **Maja Jagodic**. Thank you for giving me the chance to start my PhD in your group seven years ago. Thank you for always supporting me no matter what and always giving me the feeling that I can make this happen (even during the time when I really thought that this would never be the case). I am so happy that I got to know you and got the chance to work with you! You are amazing!!!

My co-supervisors Prof. **Fredrik Piehl** and Prof. **Bob Harris**. Thank you both for always having time to discuss or advice no matter how busy you were. I was really happy to have you both as my co-supervisors.

My almost 4th co-supervisor **Dr. Victor Guryev**. Thank you for letting me come to Groningen on several occasions, for all the patience with explaining the world of RNA sequencing data analysis to me, the table football games and ice curling. It was a lot of fun and I am really happy that we started this collaboration.

Prof. **Thomas Olsson** for forwarding my email to Maja seven years ago and by doing so giving me the opportunity to do my PhD here at CMM and to experience the international atmosphere in the Neuroimmunology Unit.

My dear colleague **Petra**. Thank you for helping me through all this and for making it easy for me to start here in STHLM. Thank you for always telling me what you think and for always knowing what to do when everything seemed completely ruined. I miss you and **Snäckan**!

Pernilla Thank you so much for everything! For your support, discussions and advice whenever needed. I will never forget the PoO, believe me!

My first student here at CMM, **Embla,** and my first master student **Eliane**. It was a pleasure and a lot of fun working with both of you.

The Epi Group **Lara, Eliane, Ewoud, Galina, Maria, Francesco.** Thank you guys for all the support, chats and laughs throughout the years.

My fellow PhDs. **Rasmus, Harald, Tojo, Sreeni, Karl, Hannes, Marie, Mathias, Susi and Melanie.** It was a pleasure to go through all of this with you together. Thanks a lot for all the advice, support, chats, laughs through all this years (7 in total ;))!

All the former fellows. **Alan, Ame, Micke, Rickard, Faiez, Mel, Nada, Roham, Shahin, Sevi, Andreas, Patrick** The same goes to you guys. You were the ones welcoming me when I started. Showing me around, without any hesitation helping me out when I think that I lost

my wallet (Roham don´t know if you remember but I do and I am still very thankful for that considering that you did not know me at all), Introducing me to Kanye West (Shahin, thank god that we did all this sorting together and that you always brought your music). Thanks to all of you. You made it very easy for me to arrive in the lab and here in STHLM!

Rux, André, Sandra and Xingmei. Thank you for all the discussions, help and advice during the last years. You made it very easy for me to start here in the lab and to find my way around.

Mohsen, Venus and Brinda. Thank you all for literally taking care of everything in the lab and your support throughout the last years and especially during the last months

Maria, Heidi and Joan. Ladies Thanks a lot for all your help throughout all the years. Maria I am still so glad that we shared this at least for me once in a lifetime Ski experience together and I am really thankful for your support during the last weeks.

Peri, Mingmei, Sunjay, Hiro and David. Thank you for all the good collaborations during the last years. Peri and Mingmei, thanks for all the advice and discussions.

Malin and Lolo. Girls, you were among the first people that I met when I arrived here in Stockholm and I am really happy about that. Thanks a lot for all the discussions and help during the last years.

Our animal house staff. **Michelle, Sandra O, Elisabeth, Joline, Linda, Helen** and all the others that I missed now but that I have worked with. To all of you: Thanks a lot for hard work and patience throughout the last years.

All the students **Sarah, Anna, Tina, Falk** that joined the EpiG-group during the last years. Thanks a lot for all the hard work during your internships. It was a pleasure to have all of you in the lab.

To all my **collaborators** and **co-authors**. Thank you for all the help and advice during the last years. Special thanks to the **ERIBA-people** in Groningen. Thank you for all your support and always making me feel very welcome in your lab!

Thanks to **all** the people working in the Unit and on the 4th floor that I forgot to mention now!

Tatiana and Anastasia for all the lovely travels and dancing evenings ☺! It was fun!

The **Research School**. **Helena** and **Jan-Alvar** thank you for organizing the school.

Steffi, Tina, Sanne, Aida, Crischi and Johanna. Ladies, it was a pleasure! I am so happy that I joined the programme, since I would have not met you guys otherwise and I would have missed out on a lot of fun, nice dinners, dancing and discussions. THANK YOU, you let STHLM become finally my 2nd home. I hope that we will always stay in touch no matter where we all end up!

Crischi, Jessi, Inka, Kinga und Dominika Mädels wat soll ich sagen… glaub wir haben allet rausgeholt aus dem Jahr hier gemeinsam in STHLM. So viele Momente wo ich einfach nur schmunzeln kann und mich wirklich freu euch getroffen zu haben. Krischi / Jessi /Kinga DANKE, für all die gemeinsamen Laufrunden die wir gedreht haben. Inka DANKE für deine Geduld mir das Kochen beizubringen. Domenica Thanks a lot for all the chats, dinners and dancing ☺! I am really happy that we shared the spinning (including really good music) experience! DANKE für all eure Unterstützung während der ganzen letzten Monate und DANKE dafür, dass ihr mich so nehmt wie ich bin. Egal wo ihr seid, ich hoffe wir schaffen es immer den Kontakt zu halten und den bisher gemeinsam erlebten Momenten noch einige hinzuzufügen! Ich bin froh, dass es euch gibt

Karine, Lina, Angeles und Natalie. Thanks a lot for all the nice lunches, discussions, pubs (Angeles for not giving up on teaching me how to dance Salsa despite my german hips that don´t want to move like they should) and support during the last months and especially during the last weeks. I am so happy that I met all of you.

Sabrina und Flo. Was hätte ich ohne euch in so manchen Situationen gemacht. Danke für die ganzen Einladungen zum Essen und eure ständige Unterstützung!

Svenja, Jule, Magda, Birhe, Jenny, Laura und Nicole. Ohne euch wäre dieses Buch nicht entstanden !! DANKE dafür, dass ihr mich die ganze Zeit ermutigt habt weiterzumachen, an meinem Traum festzuhalten und mich nicht davon abbringen zu lassen. Danke für die zahlreichen Besuche hier in STHLM! Trotz der Entfernung wart ihr immer für mich da, habt mich immer auf dem Laufenden gehalten und ich habe mich immer wieder direkt daheim gefühlt wenn ich zu Besuch im Pott war. Ich bin froh euch in meinem Leben zu haben!

Lisa, Felicitas, Alex, Svea, Evi, Kalle, Mathilda, Charlotte, Paula und ? (Jule ich weiss nicht ob ihr euch schon für einen Namen entschieden habt ;)) Es ist sooooo schön, dass es euch gibt und ich bin glücklich euch gross werden zu sehen und an euren Leben teil haben zu dürfen.

Meinen Labortussis Kristin, Jessica und Christina. Ach Mädels, ich bin so froh, dass ich mich dafür entschieden habe die Ausbildung zu machen. Ich finds super, dass ich euch getroffen hab und wir es geschafft haben über die ganzen Jahre in Kontakt zu bleiben. Danke für eure Unterstützung!

Meinen Bochumer Kadetten. Denise, Romy und Sabrina. Sooooo nun hab auch ich es fast geschafft …. Ich freu mich, dass auch wir es geschafft haben uns nicht über die Jahre zu verlieren und es immer wieder direkt so wie damals ist sobald wir uns wiedersehen. Danke auch an euch für all die Unterstützung während der ganzen letzten Jahre.

Lars. Ohne dich wäre ich nicht wo ich jetzt bin. Danke, dass du immer für mich da warst! Du fehlst mir!

Der AG Chan/Haghikia. Andrew, Aiden, Bartek, Kerstin, Isa, Björn, Simon, David und Seray. Vermiss euch immer noch! Es war eine tolle Zeit und ich hätte mir keine bessere Gruppe für meine ersten Schritte in der Wissenschaft wünschen können. Danke für eure Unterstützung während der ganzen Jahre! Es ist immer wieder schön euch zu sehen oder von euch zu hören.

Prof. Gold. Auch an Sie ein großes DANKE für eine tolle Zeit in Bochum und all die Unterstützung während der letzten Jahre.

Frau Reppel. Es hätte so einiges schief gehen können wenn ich Sie nicht während meine ersten Schuljahre an meiner Seite gehabt hätte. Danke dafür, dass Sie uns den Einstieg in den "Ernst des Lebens" so einfach gemacht haben und die Weichen in eine gute Richtung gestellt haben!

Bastian. Danke für stetiges Feedback und studenlanges Zuhören! Ich bin froh dich zu meinen Freunden zählen zu dürfen.

Oma und Opa. Ihr habt soviel zu diesem Buch beigetragen. Danke für alles! Ich vermisse und umarme euch!

Mama und Papa. Ich bin so froh, dass es euch gibt! Danke für eure bedingunglose Unterstüzung während der ganzen letzten Jahre!!! Hab euch lieb!

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